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C9orf72 hexanucleotide repeat expansions in Chinese sporadic amyotrophic lateral sclerosis

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ABSTRACT

A hexanucleotide repeat expansion (HRE) in the *C9orf72* gene has been identified as the most common mutation in amyotrophic lateral sclerosis (ALS) among Caucasian populations. We sought to comprehensively evaluate genetic and epigenetic variants of *C9orf72* and the contribution of the HRE in Chinese ALS cases. We performed fragment-length and repeat-primed polymerase chain reaction to determine GGGGCC copy number and expansion within the *C9orf72* gene in 1092 sporadic ALS (sALS) and 1062 controls from China. We performed haplotype analysis of 23 single-nucleotide polymorphisms within and surrounding *C9orf72*. The *C9orf72* HRE was found in 3 sALS patients (0.3%) but not in control subjects ($p = 0.25$). For 2 of the cases with the HRE, genotypes of 8 single-nucleotide polymorphisms flanking the HRE were inconsistent with the haplotype reported to be strongly associated with ALS in Caucasian populations. For these 2 individuals, we found hypermethylation of the CpG island upstream of the repeat, an observation not detected in other sALS patients ($p < 10^{-8}$) or controls. The detailed analysis of the *C9orf72* locus in a large cohort of Chinese samples provides robust evidence that may not be consistent with a single Caucasian founder event. Both the Caucasian and Chinese haplotypes associated with HRE were highly associated with repeat lengths >8 repeats implying that both haplotypes may confer instability of repeat length.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by loss of upper and lower motor neurons. The disorder occurs as sporadic ALS (sALS) in most cases, while 5%–10% of cases are familial (fALS).

Because *SOD1* mutations were linked to fALS in 1993 (Rosen et al., 1993), a number of causative genes have been identified. A large hexanucleotide (GGGGCC) repeat expansion (HRE) in the first intron of the *C9orf72* gene has been identified as the most common mutation detected in ALS and frontotemporal dementia patients in Caucasian populations (DeJesus-Hernandez et al., 2011; Millecamps et al., 2012; Renton et al., 2011). Mutations in these genes may also be found in sALS, but other than *C9orf72* HRE each accounts for <1% of cases (Renton et al., 2014).

The *C9orf72* HRE mutation has been comprehensively screened and assessed in Caucasian populations. The HRE frequency amongst sALS cases ranges from 3.2% to 21% in different populations (Beck et al., 2013; Galimberti et al., 2014; Majounie et al., 2012; Ratti et al., 2012; Renton et al., 2011). However, in Asian sALS, it was found to be rare in Japanese cohorts (Ishiura et al., 2012; Konno et al., 2012; Majounie et al., 2012; Ogaki et al., 2012) and absent in cases from mainland China (Jiao et al., 2014; Liu et al., 2013; Majounie et al., 2012; Zou et al., 2013). Additionally, genotypes consistent with a common founder risk haplotype have been reported in most *C9orf72* HRE-carrying ALS cases in diverse populations, and it has been proposed that the HRE arose in a single common founder ~1500 years ago (Majounie et al., 2012).

The function of the protein encoded by *C9orf72* remains unclear; however, there are several hypotheses about functional consequences

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of the HRE including toxicity of the transcribed repeat, toxicity of protein dipeptides translated from the transcribed repeat, or loss of function (DeJesus-Hernandez et al., 2011; Donnelly et al., 2013; Gijselinck et al., 2012; Mori et al., 2013). Abnormal methylation of the CpG island upstream of the repeat was found in 73% of HRE carriers and was associated with downregulation of *C9orf72* transcripts (Xi et al., 2013).

To date, large cohorts of Caucasian ALS cases have been assessed for the *C9orf72* HRE, however, comparatively few studies of small cohorts of Asian cases have been conducted. We aimed to more accurately determine the frequency of the *C9orf72* HRE in a large cohort of Chinese sALS subjects and investigate its relationship with single-nucleotide polymorphism (SNP) haplotypes and nearby CpG methylation.

2. Materials and methods

2.1. Participants

Patients attending the ALS specialty clinic at the Department of Neurology of the Peking University Third Hospital, Beijing, China, from 2003–2013 were recruited. Patients in the case cohort were diagnosed with ALS according to El Escorial revised criteria (Brooks et al., 2000) by a neurologist specializing in ALS. In the present analysis, only sALS cases were included based on self-report and clinical interview. The cohort consisted of 65% males, while mean age of onset of sALS was 49.7 ± 12.2 years. The proportion of patients with bulbar onset was 17%. The control samples were from individuals who attended the same hospital and Shanghai Changzheng Hospital and who had no medical or family history of neurological disorders. All patients and control subjects were from mainland China and of Chinese origin. Control cohorts were age- and sex-matched neurologically healthy individuals. Table 1 describes the demographic characteristics of the cases and controls. All patients and controls provided written informed consent for the clinical and genetic studies during their visit to the neurologist. The Peking University Third Hospital and Changzheng Hospital ethics committees approved the collection of DNA samples from case and control subjects.

2.2. Repeat-primed polymerase chain reaction

Genomic DNA from 1092 patients with sALS and 1062 controls subjects was extracted from blood using standard protocols. Two-step polymerase chain reaction (PCR) was performed to detect *C9orf72* HRE as previously described (DeJesus-Hernandez et al., 2011). Briefly, fluorescent fragment-length analysis was performed with genotyping primers. The samples with a homozygous peak pattern were analyzed by fluorescent repeat-primed PCR to identify HREs. The HRE was defined as repeat number >30 (Renton et al., 2011) indicated by the typical "saw-tooth" pattern seen by repeat-primed PCR.

2.3. Haplotype SNP analysis, principal component analysis, and validation

Genome-wide genotyping was performed using Illumina HumanOmniZhongHua DNA analysis arrays (900,015 SNPs and/or individual) and manufacturer's protocols. Bead intensity data was processed and normalized for each sample and genotypes extracted in Genome Studio (Illumina). To predict haplotypes with greater accuracy, a larger cohort of 3115 control samples was used for haplotype analysis. Nine hundred thirty-nine cases and 2850 controls survived after Quality Control with 784,352 SNPs and/or individual. SNP genotypes from HRE carriers were validated by direct sequencing. The SNPs tested included 20 from the consensus risk founder haplotype in several ALS populations (Mok et al., 2012) and 3 additional SNPs flanking the repeat (previously included by Smith et al., 2013). Haplotype analysis of the 23 SNPs within and surrounding *C9orf72* was performed by direct investigation and data imputation (using IMPUTE2) (Howie et al., 2011), with the 1000 Genomes Project reference panel, phase I version 3 to determine whether the Chinese patients carried the founder haplotype associated with a risk for ALS. Haplotype reconstruction and frequency estimations were performed using PHASE (version 2.1.1, Chicago, IL, USA) (Stephens et al., 2001). Principal component analysis was conducted by using the software package EIGENSTRAT (Price et al., 2006). Detailed methods are provided in Supplementary Tables 1 and 3 in the Supplementary Materials.

2.4. DNA methylation analysis

Genome-wide methylation levels in DNA extracted from blood of 461 sALS cases and 198 controls (a subset of those with genome-wide genotyping) were measured using the HumanMethylation450 BeadChip (Illumina) following manufacturer's protocols. Bead intensity data was background-corrected and normalized using internal controls and methylation beta values extracted using the minfi package in R CRAN (http://cran.r-project.org/). Low quality samples and probes were removed. To assess the level of DNA methylation in the *C9orf72* region, beta values for all 8 probes annotated to the *C9orf72* region (based on Illumina probe annotation) were extracted, which detected 2 predicted CpG islands (From University of California Santa Cruz database) closely flanking the GGGGCC repeat.

Table 1
Demographics of the case and control cohorts analyzed for the frequency of the HRE in *C9orf72*

	Sporadic ALS patients[a]	Controls for mutation screening[b]	Controls for haplotype analysis[c]
Total	1092	1062	2850
Male	697 (65.20%)	561 (54.05%)	1213 (42.57%)
Female	372 (34.80%)	477 (45.95%)	1637 (57.43%)
Age at onset			
Mean ± SD	49.70 ± 12.22	50.25 ± 14.90[d]	—
Site of onset			
Bulbar	131 (17.08%)	—	—
Spinal	636 (82.92%)	—	—

Key: ALS, amyotrophic lateral sclerosis; HRE, hexanucleotide repeat expansion; SD, standard deviation.
[a] Data not available for the gender of 23 patients, age of onset for 311 patients, site of onset for 325 patients.
[b] Data not available for the gender for 24 control subjects, age at enrollment for 24 control subjects.
[c] Healthy controls survived after quality control.
[d] Age at enrollment for control cohort.

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